





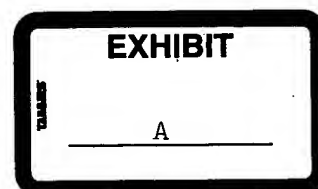
**Accumulation of Dietary Cholesterol in Sitosterolemia Caused by Mutations in  
Adjacent ABC Transporters**

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## ABSTRACT

**In normal humans, acute changes in cholesterol intake produce modest changes in plasma cholesterol levels. A striking exception occurs in sitosterolemia, an autosomal recessive disorder characterized by increased intestinal absorption and decreased biliary excretion of dietary sterols, hypercholesterolemia and premature coronary atherosclerosis. We have identified mutations in two adjacent, oppositely-oriented genes in sitosterolemia. Both genes (*ABCG5* and *ABCG8*) encode new members of the ATP-binding cassette (ABC) transporter family. The two genes are expressed at highest levels in liver and intestine and are up-regulated coordinately by cholesterol feeding. The data suggest that *ABCG5* and *ABCG8* normally cooperate to limit intestinal absorption and promote biliary excretion of sterols, and that mutated forms of these transporters predispose to sterol accumulation and atherosclerosis.**

In humans the intestine presents a barrier that prevents the absorption of plant sterols and partially blocks the absorption of cholesterol. This barrier is disrupted in the rare autosomal recessive disorder, sitosterolemia (1). Sitosterolemic patients hyperabsorb the plant sterols such as sitosterol, which provide the identifying feature of this disease (1-3). These patients also hyperabsorb cholesterol and are usually hypercholesterolemic, resulting in the development of xanthomas (cholesterol deposits in skin and tendons) and premature coronary artery disease (2,3). Unlike other forms of hyperlipidemia,

sitosterolemic subjects respond to restriction in dietary cholesterol and to bile acid resin treatment with dramatic reductions in plasma cholesterol levels (2-5).

Patients with sitosterolemia have markedly elevated (>30-fold) plasma levels of plant sterols (sitosterol, stigmasterol and campesterol) as well as other neutral sterols with modified side chains (1,6,7). Normal humans absorb only ~5% of the 200 to 300 mg of plant sterols consumed each day (8,9). Almost all of the absorbed sitosterol is quickly secreted into the bile so that only trace amounts of sitosterol and other plant sterols remain in the blood (8,9). In contrast, subjects with sitosterolemia absorb between 15 and 60% of ingested sitosterol, and they excrete only a fraction into the bile (1-4). The liver secretes sitosterol into the bloodstream where it is transported as a constituent of low density and high density lipoprotein particles (1). With the exception of the brain, the relative proportion of sterol represented by sitosterol in tissues matches that in plasma (10-25%) (10). Hyperabsorption and inefficient secretion is not limited to plant sterols. Sitosterolemic subjects absorb a higher fraction of dietary cholesterol than normal subjects, and they secrete less cholesterol into the bile (1-4). Taken together, the genetic and metabolic data indicate that sitosterolemic patients lack a gene product that normally limits the absorption and accelerates the biliary excretion of sterols (2,3).

The molecular mechanisms that limit sterol absorption are poorly understood but clues have emerged recently from studies of the orphan nuclear hormone receptor LXR (11). Mice with a targeted disruption of the LXR gene show enhanced cholesterol absorption (12). Conversely, mice treated with an LXR agonist have a marked decrease in cholesterol absorption and a corresponding increase in the intestinal expression of

mRNA encoding the ATP binding cassette protein (ABC) 1, a membrane-associated protein that has been implicated in the transport of cholesterol (11,13). We hypothesized that sitosterolemic patients might have defects in other genes that limit cholesterol absorption and that the expression of these genes would be regulated by LXR. To test this idea we used DNA microarrays to search for mRNAs that are induced by the LXR agonist T091317 in mouse liver and intestine (11,14). A transcript corresponding to a murine EST (AA237916) was induced ~2.5-fold in the livers and intestines of treated mice. This EST resembled three *Drosophila* genes that encode ABC half-transporters (*brown*, *scarlet* and *white*) expressed in the pigimentary cells of the eye (15-17). These ABC half-transporters contain six membrane spanning domains and form two types of heterodimers that transport guanine (*brown/white*) or tryptophan (*scarlet/white*). Since a human homolog of *white* (*ABCG1*) is implicated in cellular cholesterol efflux from macrophages (18,19), we reasoned that the LXR-induced protein encoded by AA237916 might be involved in sterol trafficking in liver and intestine, and hence this gene was a candidate for the defect in sitosterolemia.

A full-length cDNA corresponding to AA237916 was isolated from a mouse liver cDNA library (Origene), and this sequence was used to identify a human ortholog in the GENBANK EST database (T86384). A full-length human sequence was obtained by iterative EST database searches and by cloning from human liver cDNA libraries (Origene and Clontech). The human cDNA predicts a 651 amino acid protein (Fig. 1C) that shares 82% sequence identity with the mouse sequence (data not shown). Following the standard system of nomenclature in the ABC transporter field, we have named this

protein *ABCG5*. The amino terminal half of *ABCG5* contains the ATP-binding motifs (Walker A and B motifs) and an ABC transporter signature motif (C motif), while the carboxyl terminal region is predicted to contain six transmembrane (TM) segments (Fig. 1B) (17,20,21). A human EST clone (Unigene T93792) from *ABCG5* mapped to chromosome 2p21 between markers D2S177 and D2S119 and the map position was confirmed using a radiation hybrid panel (22). Patel and colleagues previously mapped the human sitosterolemia gene to this same region of chromosome 2 in ten independent sitosterolemic families (23).

The structure of the human *ABCG5* gene was characterized by analysis of a bacterial artificial chromosome (BAC) clone that contained the entire gene (Fig. 1A) (24). The gene spans ~28 kb and has 13 exons and 12 introns. The coding sequences and consensus splicing sequences were amplified from genomic DNA by PCR and sequenced in nine unrelated subjects with sitosterolemia (Table 1). A sitosterolemic patient from Hong Kong (proband 9) was heterozygous for a transition mutation (CGA to TGA) in codon 408 that introduced a premature stop codon between TM1 and TM2. This mutation was not present in 65 normolipidemic individuals, including 40 Chinese subjects. No other potential disease-causing mutations were identified in *ABCG5*. A transversion in codon 604 that substituted a glutamic acid for glutamine (Q604E) in the loop between TM5 and TM6 was found in five sitosterolemic probands but was also present in 23% of the alleles from normolipidemic Caucasians (n=50).

Genes encoding members of the ABC transporter family are often clustered in the genome (25). Since only a single *ABCG5* mutation was identified in our collection of

nine sitosterolemic probands, we searched the public and Celera genome sequences for other ABC transporters adjacent to *ABCG5*. An EST (T84531) that shared weak homology with the *Drosophila white* gene was identified and expanded using exons predicted by the computer program GENSCAN (26). Eleven of the 13 exons of the new gene, which we name *ABCG8*, were identified in the databases and the remaining two exons were identified by sequencing PCR-amplified cDNAs from human hepatic polyA<sup>+</sup> mRNA. *ABCG8* shares ~28% amino acid identity with *ABCG5* (Fig. 1C). Its sequence is most similar to *ABCG1*, which resembles the *Drosophila white* gene (16).

The translational start sites of *ABCG5* and *ABCG8* are separated by only 374 basepairs and the two genes are arranged in a head to head orientation (Fig. 1A). Both genes contain a translation initiation codon with an upstream in-frame stop codon. The close proximity and opposite orientation of *ABCG5* and *ABCG8* suggest that the two genes have a bi-directional promoter and share common transcriptional and regulatory elements (27,28). No obvious LXR response element was identified in the limited amount of sequence available at this time. Other gene pairs with bi-directional promoters form functional complexes (28), as may be the case for *ABCG5* and *ABCG8*.

The predicted intron-exon boundaries of human *ABCG8* were confirmed by DNA sequencing. The single strand conformation polymorphism technique was used to screen the exons and flanking intron sequences of *ABCG8* in the nine sitosterolemic subjects (Table 1) (29,30). DNA sequencing of abnormally-migrating fragments revealed six different mutations (Table 1 and Fig. 1B). The first patient to be described with sitosterolemia (proband 1) was homozygous for a nonsense mutation (c.1083G>A) in

exon 7 (Fig. 1B) that introduced a premature termination signal codon at codon 361, terminating the protein prior to TM1 (1). Three other unrelated Caucasian sitosterolemic subjects (probands 3, 5 and 8) were heterozygous for the same mutation (3,31,32). One of these probands (proband 5) were originally given the diagnosis of pseudohomozygous familial hypercholesterolemia, an autosomal recessive disorder characterized by hypercholesterolemia, tendon xanthomas and exquisite sensitivity to dietary cholesterol (5,32). Many of the patients originally diagnosed with pseudohomozygous FH were subsequently found to have sitosterolemia, as was the case with this patient and proband 6 (5,32). Proband 3 was heterozygous for another nonsense mutation in exon 13 that introduced a stop codon 15 residues from the carboxyl terminus of *ABCG8*. The resulting protein would lack part of the last predicted transmembrane domain, and the short cytoplasmic domain, which contains a cluster of positively charged residues that may be important in positioning these proteins in the membrane (33).

Two missense mutations identified in *ABCG8* produced nonconservative amino acid changes in residues that are conserved between the humans and mouse proteins (data not shown) as well as in *ABCG5*. One Chinese patient (proband 4) was heterozygous for a missense mutation in exon 6 in codon 263 (R263Q). An Amish subject with sitosterolemia was homozygous for a missense mutation (G574R) in a residue that is conserved in mouse and human *ABCG8*. Genomic DNA was available from an additional three of the four living affected family members in this large Amish pedigree (34,35) and these individuals were homozygous for this same missense mutation (data not shown). A third nonconservative missense mutation was an arginine substitution for

a leucine in codon 596. The corresponding sequence in *ABCG5* is another nonpolar amino acid, glutamine. None of these three missense mutations were identified in 100 alleles from ethnically-matched normolipidemic subjects, which is consistent with these being disease-causing mutations. A common polymorphism (Y54C) with an allele frequency of 23% in control subjects (n=100 alleles) was also identified in *ABCG8*.

Thus, we identified two mutant alleles at the *ABCG8* locus in four of the nine sitosterolemic patients. Four patients had a single mutant allele in *ABCG8* and one patient had a single mutant allele in *ABCG5*. The identification of multiple different *ABCG8* mutations in subjects with sitosterolemia, including nonsense mutations that appear incompatible with protein function, provides strong evidence that sitosterolemia is caused by defects in this gene. It also seems likely that the mutation we found in *ABCG5* causes sitosterolemia, although the identification of additional mutations in this gene will be required to substantiate this deduction. It is possible that some mutations causing sitosterolemia were not detected by SSCP or because they were located in sequences not screened in this study, including regulatory sequences or those required for mRNA processing. Alternatively, it remains possible that mutations in another gene (perhaps a different ABC transporter) within the genomic interval mapped by Patel *et al.* (23) can cause sitosterolemia when present in combination with mutations in *ABCG5* or *ABCG8*.

To determine if *ABCG5* and *ABCG8* are regulated coordinately, we examined the tissue distributions of their mRNAs in humans and mice, and their responses to cholesterol feeding in mice. In humans, liver was the major site of expression of both genes (Fig. 2A). For both *ABCG5* and *ABCG8*, one major transcript predominated but

other mRNAs were visible by RNA blotting. Additional studies will be required to determine the identity of these transcripts, which presumably result from alternative splicing of differential polyadenylation. RNAs of the same sizes, as those seen in the liver were seen in the small intestine on a longer exposure times (data not shown). Transcripts for both *ABCG5* and *ABCG8* could be PCR-amplified from human intestinal mRNA, but not from human cholesterol-loaded differentiated monocytes (THP-1 cells) (data not shown). In mice, *Abcg5* and *Abcg8* were expressed at higher levels in the intestine than in the liver, although the relative amounts of expression in these two tissues may be strain- and sex-specific (data not shown). Inasmuch as the expression of these two genes is regulated by dietary sterols (see below), definitive studies of tissue expression in humans will require careful control of dietary intake.

If *ABCG5* and *ABCG8* protect against the accumulation of sterols, then their expression levels would be predicted to increase with cholesterol feeding. To test this hypothesis, mice were fed a high cholesterol diet (2%) and killed after 1, 7 or 14 days. The mRNA levels of both genes increased ~2-fold in intestine and much more markedly in liver within one week of initiation of the high cholesterol diet (Fig. 2B). These changes were maintained at two weeks (data not shown). As expected, the plasma levels of cholesterol did not significantly change in the cholesterol-fed mice (from 95 mg/dl versus 93 mg/dl) since mice rapidly and efficiently convert dietary cholesterol into bile acids and excrete both cholesterol and bile acids into the bile (36). LXR plays a central role in this regulated process by increasing the expression of multiple hepatic genes that promote bile acid synthesis and biliary secretion (11). The ligands for LXR include

hydroxylated sterols that are derived from cholesterol (37,38). Since *ABCG5* is induced by an LXR agonist, it is possible that dietary sterols induce these genes through LXR.

The aforementioned data indicate that *ABCG5* and *ABCG8* are putative ABC half-transporters that may partner to generate a functional protein. The juxtaposition of these two genes on chromosome 2, the coordinate regulation of their mRNAs in the liver and intestine with cholesterol feeding, and the observation that mutations in either gene are associated with sitosterolemia, suggest that these two proteins form a functional complex that mediates efflux of dietary cholesterol from the intestine, and thus protects humans from sterol overaccumulation. This protection is especially important in Western societies that consume high cholesterol diets. Loss of function of these proteins causes sitosterolemia. It seems possible that subtle defects in these proteins or in their regulation may underlie the variable responses of individuals to high cholesterol diets.







35. T. H. Beaty, *et al.*, *Am. J. Hum. Genet.* **38**, 492 (1981).
36. D. W. Russell, K. D. Setchell, *Biochem.* **31**, 4737 (1992).
37. B. A. Janowski, *et al.*, *Nature* **383**, 728 (1996).
38. B. A. Janowski *et al*, *Proc. Natl. Acad. Sci.* **96**, 266 (1999).
39. V. Jokinen, *et al.*, *J. Biol. Chem.* **269** 26411 (1994).
40. We wish to thank Tommy Hyatt, Yuanlan Liao, Lisa Beatty, Bill P. Crider, Donna Virgil, Ross Wilson, Sijing Niu, Jie Wu, Sindhu Padmanabhan and Miki Rich for excellent technical assistance; the physicians who provided tissue samples from patients with sitosterolemia, including, Drs. Thomas DiSessa, Antonio Gotto, John Kane, L. C. K. Low, and E. R. Nye; Drs. Michael S. Brown, and Joseph L. Goldstein for making these sample materials available to us and for helpful discussions; We also want to thank David Russell for helpful discussions. This work was supported by the National Institute of Health (HL20948), the W. M. Keck and the W. R. Reynolds Foundations. K. E. B. is supported by the Norwegian Research Council and G. G. is supported by a Training Grant in Cardiovascular Research (HL07360).





of the cDNA was amplified and the fragment radiolabeled (Megaprime DNA Labeling System, Amersham) prior to incubation with the blot in Rapid-hyb buffer ( $1 \times 10^6$  cpm/ml) (Amersham). The blot was washed and subjected to autoradiography for 18 h using Kodak X-OMAT-blue film (39). The results were identical when probes generated from the 3' untranslated regions of both cDNAs were used. **(B)** Cholesterol feeding-induces coordinate increases in levels of *ABCG5* and *ABCG8* mRNA. Seven-week-old male mice (129S3/SvImj) were fed powdered chow (Harlan Teklad Rodent Diet) in the absence or presence of cholesterol (2%, w/v). Mice were killed after one or seven days in the light phase of the cycle. Total RNA was isolated using RNA-STAT (TelTest) from the liver and three equal segments of the small intestine (duodenum, jejunum and ileum). The tissue RNAs were pooled from three animals and aliquots (15  $\mu$ g) used to make duplicate northern blots (30). The mouse cDNAs for *ABCG5* and *ABCG8* were used as probes. Cyclophilin was used as an internal standard. The results were identical when probes generated from the 3' untranslated regions of both cDNAs were used.





-----Original Message-----

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Here are the human cDNA sequences from human brown (ABCG5) and ABCG8 (which we call Goldstein) attached. Let me know if these is anything else you need.

Helen Hobbs

